Appendix A

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Molecular Characterization and Distribution of Genes Encoding Members of the Type III Effector NleA Family among Pathogenic *Escherichia coli* Strains[▽]

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In this study, we investigated the occurrence of the previously described gene *nleA*$_{4795}$ and variants of *nleA*, putatively encoding non-locus-of-enterocyte-effacement-encoded type III effector proteins with functions that are unknown. *nleA* variants were detected in 150 out of 170 enteropathogenic *Escherichia coli* strains and enterohemorrhagic *E. coli* strains, two of them being *eae* negative. Besides the known variants *nleA*$_{4795}$, Z6024, and the *espI*-like gene, 11 novel *nleA* variants with different lengths and sequence identities at the deduced amino acid level (between 71% and 96%) have been identified. Whereas most of the serogroups associated with more severe disease were quite homogenous with respect to the presence of a particular *nleA* variant, other serogroups were not. Moreover, Southern blot hybridization revealed that certain strains carry two copies of *nleA* in their chromosome, frequently encoding different variants. In most cases, the open reading frame of one of the copies was disrupted, usually by an insertion element. Furthermore, transmission of the type III effector-encoding gene could be shown by transduction of *nleA*-carrying bacteriophages to a laboratory *E. coli* strain.

Enterohemorrhagic *Escherichia coli* (EHEC) and enteropathogenic *E. coli* (EPEC) can cause serious gastrointestinal diseases and are able to damage the gut epithelia of their hosts by a sophisticated mechanism of attachment and effacement (11). Following adherence to intestinal cells, attaching and effacing (A/E) *E. coli* organisms interfere with cytoskeletal processes and produce a specific histopathological feature that is characterized by localized destruction of the brush border microvilli and intimate adhesion of the bacteria to the plasma membrane of the host cells (21). The development of A/E lesions is mediated by a type III secretion system (T3SS), which is able to translocate effector proteins via a needle complex directly in the cytoplasm of host cells (18). The machinery of this secretion system and its effector proteins are located within the bacterial chromosome on a pathogenicity island termed the locus of enterocyte effacement (LEE) (14).

It has been shown that additional effector proteins encoded by genes outside the LEE in cryptic or intact prophages are translocated by the LEE-encoded T3SS. The majority of these effectors have been identified by a proteomics approach with the mouse A/E pathogen *Citrobacter rodentium* (8) as well as by using bioinformatics, proteomics, and translocation assay approaches with the *E. coli* O157:H7 strain RIMD 0509952 (37). This group of non-LEE-encoded effectors also includes Cif (24), NleA/EspI (16, 27), TccP/EspF$_U$ (3, 15), EspJ (7), NleB (20), and EspK (38). The cycle-inhibiting factor Cif blocks the cell cycle at the G$_2$/M-phase transition and is involved in the formation of stress fibers (24). The Tir cytoskeleton coupling protein TccP/EspF$_U$ binds N-WASP and leads to Nck-independent actin polymerization (3, 15). EspJ may play a role in host survival and pathogen transmission (7). NleB is probably a virulence determinant (20), whereas EspK could be involved in intestinal colonization (38).

The non-LEE-encoded effector NleA/EspI of *C. rodentium* shows 81% identity at the amino acid level to the protein Z6024, encoded by phage CP-933P in *E. coli* O157:H7 strain EDL933 (30), and 78% and 76% identity, respectively, to NleA$_{4795}$, which is encoded by the Stx1-converting prophage BP-4795 of *E. coli* O84:H4 strain 4795/97 (6), and the EspI-like protein, encoded together with Cif by a prophage in the genome of the rabbit EPEC O103:H2 strain E22 (24). The non-LEE-encoded effectors NleA and NleA$_{4795}$ localize close to the Golgi apparatus of HeLa cells (6, 16). Moreover, experiments with a mouse model showed that NleA/EspI is necessary for virulence (16, 27), but the function of this effector protein is still unknown. Mundy et al. (26) examined 232 EPEC and 93 EHEC strains for the presence of *espI* using colony hybridizations. They could detect *espI* in 53% of the LEE-positive EPEC strains tested. In *eae*-positive EHEC isolates, it was found more frequently. Consequently, 37 of 43 (86%) LEE-positive EHEC strains contained *espI*, and the authors assumed a correlation between the presence of *espI* and certain intimin subtypes in EPEC strains. However, it was not possible to define such an association for the occurrence of *espI* and a specific intimin type in EHEC strains. Furthermore, they could detect *espI* more commonly in strains from patients suffering from a more severe disease (26).

The aim of the present study was to determine the distribution of *nleA*$_{4795}$ and related variants among pathogenic intestinal *E. coli* strains. Furthermore, we were interested in a

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TABLE 1. PCR primers, cycling conditions, and PCR product lengths

Primer for *nleA* target gene variants	Primer nucleotide sequence[a]	PCR condition		Length of PCR product (bp)
		Annealing temp (°C)	Elongation time (s)	
V83-for2	5′-ACAGCAACATGCACCGGAATGC-3′	58	90	9,59–1,112
V83-rev2	5′-CTTCCATCGCACGTATATCAGC-3′			
V83-for2	5′-ACAGCAACATGCACCGGAATGC-3′	55	90	1,015–1,168
V83-rev3	5′-GATATCGATGACCACATCTTCAGG-3′			
VarA-for*	5′-TATTAAAGCTGTCCACATCGG-3′	50	120	1,434–1,584
VarA-rev*	5′-TGGTGTATTTGTTTTGTGGGG-3′			
VarA-for*	5′-TATTAAAGCTGTCCACATCGG-3′	50	120	1,333–1,483
VarA-rev2*	5′-AGCTTAGACTCTTGTTTCTCG-3′			

[a] Primers designed to amplify *nleA* and its variants. Asterisks in first column indicate primers used for cycle sequencing reactions for amplification and sequencing of the whole ORF.

possible association and correlation of the presence of *nleA* variants with serotypes and *eae* types.

MATERIALS AND METHODS

Bacterial strains. The 170 bacterial strains used in this study mainly were taken from our strain collection. A large set of strains was isolated during routine diagnostic work in the laboratory of Helge Karch at the Institute of Hygiene and Microbiology, University of Würzburg, Germany, in the years 1977 to 2001. Other strains were provided by colleagues during the European Union project QLK-2-20060, and the sequences of some of these strains already have been published (1). Strains with the prefix CB originate from Lothar Beutin, Federal Institute for Risk Assessment, Berlin, Germany. Most of the *E. coli* O84 strains were a gift of Helmut Tschäpe, Robert-Koch Institute, Wernigerode, Germany, and strain S21195 was provided by Ulrich Busch, Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit, Oberschleiβheim, Germany. The *E. coli* O103:H2 strain UTI was donated by Phil I. Tarr, Washington University School of Medicine, St. Louis, MO. Other strains included in this study were H.I.8 (13), E2348/69 (22), RDEC-1 (4), EDL933 (28), PMK5 (25), CF11201 (10), CL37 (19), and 95NR1 (39). Serotype, origin, and disease association of the strains are described in Table 2. The *E. coli* K-12 strain C600 was used as a control in different experiments, and the *E. coli* K-12 strain C600, harboring plasmid pK18 (31), was used in the transduction experiments.

Molecular techniques. Amplification of Shiga toxin genes (*stx*) and characterization of stx_1, stx_2, and stx_{2f} were carried out by PCR as described previously (5, 23, 35, 36). Detection of *eae* and its subtypes ι, κ, θ, and ζ was performed as described previously (33, 40). The PCR protocols for the specific detection of other *eae* subtypes have been modified by increasing the annealing temperature to 67°C (for β and ε), 52°C/62°C (for γ), and 58°C (for η) (29, 34, 40).

The amplification of *nleA* variants for subsequent restriction was performed in a total volume of 70 μl. Other PCRs were carried out in a total volume of 50 μl containing 5 to 6 μl of bacterial suspension and containing one to three single colonies in 150 mM NaCl, 200 μM of each deoxynucleoside triphosphate, 30 pmol of each primer, 1× *Taq* polymerase buffer, and 3 U of *Taq* DNA polymerase (Genaxxon Bioscience). PCR started with an initial denaturation for 5 min at 94°C, followed by 30 cycles of amplification, consisting of denaturation for 30 s at 94°C, annealing for 60 s at a specific temperature (Table 1), and elongation at 72°C for a certain time course (Table 1). After the last cycle, a final elongation step of 5 min at 72°C completed the reaction.

Restriction of PCR products was performed, according to the manufacturer's recommendations, with BseNI and PstI (Fermentas). For differentiation of *nleA8-1* from *nleA3* and *nleA4*, the restriction endonucleases BclI, NheI, and SphI were used, and the variants *nleA1*, *nleA2*, and the *espI*-like gene were distinguished by restriction with Bpu1102I and CseI.

For DNA sequencing, *nleA* variants were amplified with primer pairs VarA-for/VarA-rev and VarA-for/VarA-rev2. To separate mixed PCR products, the respective product was extracted either from a gel using the QIAquick gel extraction kit (QIAGEN) or by being cloned into the pCR2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen) according to the manufacturers' instructions. DNA sequencing was performed with the CEQ 8000 genetic analysis system (Beckman Coulter) using the CEQ dye terminator cycle sequencing quick-start kit (Beckman Coulter) by following the manufacturer's recommendations. The sequences obtained from the raw data were edited and analyzed with BioEdit (17).

Preparation of genomic DNA. For preparation of genomic DNA, 2 ml of an overnight culture was centrifuged for 10 min at 6,000 rpm, resuspended in 1 ml 0.9% NaCl solution, and centrifuged again for 5 min at 13,000 rpm in a bench-top centrifuge. The bacterial pellet was resuspended in 400 μl STET buffer (233 mM sucrose, 50 mM Tris-HCl [pH 8.0], 20 mM EDTA [pH 8.0], 5% [vol/vol] Triton X-100, and 1.8 mg/ml lysozyme), incubated for 5 min at room temperature, heated for 1 min to 100°C, and immediately cooled on ice for 2 min. After this procedure, 30 μl of 10% (wt/vol) sodium dodecyl sulfate and 3 μl proteinase K (20 mg/ml) (Carl Roth) were added, and the solution was incubated for 1 h at 56°C. After the addition of 3 μl RNase A (100 mg/ml) (Sigma-Aldrich) and 200 μl distilled water, the mixture was incubated for another 30 min at 37°C. Finally, after extraction with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), the liquid phase was transferred into a fresh tube. The genomic DNA was precipitated with 2.5 volumes of ethanol and a 1/10 volume of sodium acetate (pH 7.2) for 30 min at −20°C, followed by centrifugation for 30 min at 13,000 rpm and 4°C. The pellet was washed with 70% ethanol and dissolved in 100 μl distilled water.

Southern blot hybridization. Ten micrograms of genomic DNA was digested overnight with MluI (Fermentas), separated on a 0.8% agarose gel, and transferred to a nylon membrane by vacuum blotting (32). Southern blot hybridization was performed with the DIG DNA labeling and detection kit (Roche Diagnostics) by following the manufacturer's recommendations. The hybridization was conducted at 68°C overnight using an $nleA_{4795}$ probe. This probe was generated from *E. coli* strain 4795/97 by PCR with the primer pair V83-for2/V83-rev3 after purification of two gel extracts with the QIAEX II gel extraction kit (QIAGEN) and was labeled with the Klenow fragment of the DNA polymerase included in the DIG DNA labeling and detection kit (Roche Diagnostics). For identification of the size of the obtained DNA fragments, a GeneRuler 1-kb DNA ladder and λ-mix marker 19 (Fermentas) were used.

Phage transduction. An overnight culture of the respective *E. coli* strain was used to inoculate 200 ml of Luria-Bertani (LB) broth containing 1 ml 1 M $CaCl_2$, followed by an incubation with vigorous shaking until an optical density at 600 nm of 0.8 was obtained. After adjusting the culture with 0.05 to 0.25 μg/ml norfloxacin (25 mg/ml in glacial acetic acid) and addition of 1 ml 1 M $CaCl_2$, the flask with the bacterial suspension was incubated overnight. The phage particles were separated from the cell debris by centrifugation (7,500 × *g*, 30 min, 4°C), followed by filtration through a funnel filter (Whatman). To remove bacterial nucleic acids, DNase I and RNase A (Sigma-Aldrich) were added to final concentrations of 0.5 μg/ml each. After incubation at 37°C for 45 min, sodium chloride was added to a final concentration of 5.8% (wt/vol) and was dissolved, and the solution was incubated on ice for 1 h. After a centrifugation step (7,500 × *g*, 10 min, 4°C), the phage particles were precipitated by adjusting the supernatant to 10% (wt/vol) polyethylene glycol 6000. After polyethylene glycol 6000 was dissolved at room temperature, the mixture was incubated on ice for 1 h. Phage particles were harvested by centrifugation (9,500 × *g*, 30 min, 4°C). The resulting phage pellet was dried at room temperature and dissolved in 1 ml of SM buffer (100 mM NaCl, 8 mM $MgSO_4 \cdot 7H_2O$, 50 mM Tris-HCl [pH 8], and 0.01% [wt/vol] gelatin).

For transduction, 100 μl of different dilutions of the obtained phage lysate was mixed with 100 μl bacterial culture of *E. coli* C600/pk18 (optical density at 600 nm, ~0.8) and 2 μl 1 M $CaCl_2$. The culture was incubated for 4 h at 37°C without shaking. Afterwards, 4 ml LB medium, 20 μl 1 M $CaCl_2$, and 50 μg/ml kanamycin were added, followed by an incubation for 48 h at 37°C and shaking at 180 rpm.

TABLE 2. Distribution of *stx* types, *eae* subtypes, and *nleA* variants among 170 pathogenic *E. coli* isolates and restriction fragment lengths from Southern blot hybridizations with an *nleA*$_{4795}$ probe

Serotype (no of isolates)	Host	Disease[a] (no. of cases)	*stx* type	*eae* type	*nleA* variant(s)	Hybridization product size(s) in kb (no. of products)
O15:H− (1)	Rabbit	D		β	Z6024	
O26:H11 (1)	Human	NK	1	β	Z6024,[b] *nleA8-1*	8.6, 11.8
O26:H11 (3)	Human	HUS	2	β	Z6024,[b] *nleA8-1*	12, 24 (2)
O26:H11 (2)	Human	HUS (1), NK (1)	2	β	*nleA8-1*	25
O26:H11 (2)	Human	HUS (1), D (1)	1	β	Z6024,[b] *nleA8-1*	12, 24
O26:H− (1)	Human	NK	1/2	β	Z6024,[b] *nleA8-1*	8.6, 11.8
O26:H− (2)	Human	D (1), AS (1)	1	β	Z6024,[b] *nleA8-1*	12, 24
O26:H− (2)	Human	NK (1), AS (1)	2	β	Z6024,[b] *nleA8-1*	12, 24
O26:H− (5)	Human	HUS (4), D (1)	2	β	Z6024,[b] *nleA8-1*	12, 24
O49:H2 (3)	Human	D		β	*espI*-like gene	20 (2)
O49:H2 (1)	Human	D		β		No signal
O49:H2 (1)	Rabbit	NK		β	*espI*-like gene	4.3
O49:H10 (1)	Dog	NK		κ	Z6024	9
O49:H18 (1)	Pig	NK		κ	*nleA2*	16
O49:H35 (1)	Cattle	NK		κ	*nleA2*	17
O49:NM (1)	Human	HC		κ	*nleA10*	4.8
O49:NM (1)	Cattle	NK		κ		No signal
O49:NM (1)	Dog	D				No signal
O49:NM (3)	Pig	NK		β	*nleA8-1*, *nleA8-1*	4.5, 23
O84:H2 (1)	Cattle	NK	1	ζ	*nleA4*	24
O84:H2 (1)	Human	D	1	ζ	*nleA9*, *nleA8-2*[c]	7, 30
O84:H2 (1)	Human	NK	1	ζ	*nleA9*, *nleA8-2*[c]	30
O84:H2 (1)	Human	D	1	ζ	*nleA7*, *nleA8-2*[c]	6.8, 30
O84:H4 (1)	Human	D	1	ζ	*nleA*$_{4795}$	11
O84:H28 (2)	Human	NK	1	ζ	*nleA*$_{4795}$	11 (1)
O84:H− (1)	Human	D	1	ζ	*nleA9*, *nleA8-2*[c]	30
O84:H− (1)	Human	D	1	ζ	*nleA7*, *nleA8-2*[c]	6.8, 30
O84:H− (2)	Cattle/sheep	NK	1	ζ	*nleA7*, *nleA8-2*[c]	6.8, 30
O84:H− (1)	Pig	NK		θ	*nleA8-2*, *nleA8-1*[b]	13, 25
O84:nt (1)	Human	D	1	ζ	*nleA9*	30
O84:nt (2)	Human	D (1), NK (1)	1	ζ	*nleA7*, *nleA8-2*[c]	6.8, 30
O84:nt (1)	Human	D	1	ζ	*nleA9*, *nleA8-2*[c]	7, 30
O103:H2 (2)	Rabbit/chicken	D		β	*espI*-like	13
O103:H2 (1)	Meat		1	ε	*espI*-like	24.5
O103:H2 (1)	Human	SIDS		ε	*espI*-like	24.5
O103:H2 (1)	Human	D		β	*espI*-like, *nleA11*	7, 24.5
O103:H2 (3)	Human	HUS	2	ε	*espI*-like	24.5
O103:H2 (3)	Human	NK (2), AS (1)	1	ε	*espI*-like	24.5 (3)
O103:H2 (1)	Human	NK	1		*espI*-like	24.5
O103:H2 (4)	Human	HUS (3), UTI (1)	1	ε	*espI*-like	24.5 (3)
O103:H7 (1)	Dog	HC				No signal
O103:H11 (1)	Human	NK	1	β	*nleA8-1*	25
O103:H18 (1)	Human	D	1	ε	*espI*-like	24.5
O103:H− (2)	Human	HUS (1), D (1)	1	ε	*espI*-like	24.5 (1)
O103:H− (1)	Human	HUS	2	ε	*espI*-like	24.5
O111:H2 (1)	Human	NK		β	*espI*-like	25
O111:H2 (1)	Human	NK		θ	Z6024	11.2
O111:H2 (2)	Human	D	1	θ	*nleA8-1*	27 (1)
O111:H8 (1)	Human	HUS	1	θ	*nleA8-1*	27
O111:H− (1)	Human	NK		β	*espI*-like	
O111:H− (6)	Human	HUS (2), D (4)	1	θ	*nleA8-1*	27 (4)
O111:H− (7)	Human	HUS	1/2	θ	*nleA8-1*	27 (2)
O111:H− (1)	Human	HUS	1/2		*nleA8-1*	27
O118:H5 (1)	Human	NK		κ	Z6024	
O125:H− (1)	Human	D		η	*nleA8-2*	12
O127:H6 (1)	Human	NK		α		No signal
O128:H2 (3)	Human	D (2), AS (1)	1/2			No signal (3)
O128:H2 (7)	Human	D		β	*espI*-like	20 (3)
O128:H2 (2)	Rabbit/chicken	D (1), AS (1)		β	*espI*-like	20
O128:H2 (1)	Sheep	AS	1/2			No signal
O128:H2 (2)	Pigeon	AS	2f	β	*espI*-like	10 (1)
O128:B12 (1)	Human	D	2f	β	*espI*-like	10
O128:H− (1)	Human	D	1			No signal
O128:H− (2)	Human	D (1), AS (1)	1/2			No signal (2)
O145:H4 (1)	Human	NK		ι		No signal

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TABLE 2—*Continued*

Serotype (no of isolates)	Host	Disease[a] (no. of cases)	*stx* type	*eae* type	*nleA* variant(s)	Hybridization product size(s) in kb (no. of products)
O145:H28 (1)	Human	HUS	2	γ	*nleA3*	5
O145:H28 (2)	Human	D	1	γ	*nleA5*	5.5
O145:H28 (1)	Human	HUS	2	γ	*nleA6-1*	9.8
O145:H28 (1)	Human	D				No signal
O145:H28 (1)	Pig	AS		γ	*nleA11*	19
O145:H34 (1)	Human	D		ι		No signal
O145:NM (6)	Human	HUS (5), D (1)	2	γ	*nleA2*	30 (2)
O145:NM (2)	Human	HUS (1), D (1)	1	γ	*nleA2*	30 (2)
O145:NM (1)	Cattle	AS	1	γ	*nleA2*	30
O145:NM (1)	Pig	NK		γ	*nleA2*	
O145:NM (1)	Human	HUS	2	β	Z6024	31
O145:NM (1)	Human	HUS	1/2	γ	*nleA2*	30
O156:H1 (1)	Human	AS		ζ	1	6
O156:H8 (4)	Human	NK (3), AS (1)		θ		No signal (4)
O156:H8 (2)	Pig	NK (1), D (1)		θ	Z6024	9 (1)
O156:H21 (1)	Human	D	1/2			No signal
O156:H21 (1)	Cattle	NK	1	ζ	*nleA3*, *nleA8-2*[c]	4.8, 6.2
O156:H25 (1)	Human	AS	1	ζ	*nleA3*, *nleA8-2*[c]	4.8, 6.2
O156:H25 (1)	Human	NK		θ	*nleA6-2*, *nleA8-2*[c]	6.2, 9.8
O156:H25 (1)	Human	NK		ζ	*nleA6-1*, *nleA8-2*[c]	6.2, 9.8
O156:H25 (1)	Cattle	NK		ζ	*nleA3*, *nleA8-2*[c]	5.5, 6.2
O156:H25 (4)	Sheep	NK	1	ζ	*nleA6-1*, *nleA8-2*[c]	6.2, 11.5 (4)
O157:H7 (1)	Human	D		γ	Z6024	10
O157:H7 (1)	Ground beef		1/2	γ	Z6024	12
O157:H7 (4)	Human	HUS (3), AS (1)	1/2	γ	Z6024	12 (1)
O157:H7 (4)	Human	HUS (2), D (2)	2	γ	Z6024	12 (2)
O157:H− (1)	Human	NK		γ	Z6024	
O157:H− (2)	Human	D (1), AS (1)	1/2	γ	Z6024	12 (2)
O157:H− (1)	Human	E	1/2	γ	Z6024	
O157:H− (2)	Human	HUS	2	γ	Z6024	9 (1), 12 (1)
O157:H− (4)	Human	HUS (3), AS (1)	2	γ	Z6024	

[a] NK, not known; D, diarrhea; AS, asymptomatic; HC, hemorrhagic colitis; SIDS, sudden infant death syndrome; UTI, urinary tract infection; E, enteritis.
[b] Variant gene with a 1-bp deletion of either the Z6024 or *nleA8-1* sequence.
[c] The 5′ end of *nleA8-2* was missing from this gene.

The culture then was centrifuged for 30 min at 3,500 × *g* and at 4°C, and the pellet was plated on LB agar containing 50 μg/ml kanamycin.

Nucleotide sequence accession numbers. The coding sequences of the variants *nleA1* to *nleA11* have been deposited in the GenBank database under continuous accession numbers AM421995 to AM422007.

RESULTS

Distribution of *nleA* variants in EHEC and EPEC strains. One hundred seventy EHEC and EPEC strains, which were predominantly human stool isolates of serogroups O26, O103, O111, O145, and O157 and were associated with severe human disease, as well as animal and human isolates of serogroups O49, O84, O128, and O156, with minor roles in human pathogenicity, were selected for this study. The 135 human isolates originated from asymptomatic carriers and patients with symptoms ranging from diarrhea to hemolytic-uremic syndrome (HUS), which were chosen to assess the occurrence of associations with more severe disease as postulated by Mundy et al. (26). Another 33 strains originated from feces of animals, and 2 were food isolates. The strains mainly were isolated in Germany, other European countries, the United States, Canada, Brazil, and Australia. Mundy et al. (26) detected *nleA* only in association with the intimin-encoding gene *eae*. Therefore, we included 157 *eae*-positive strains. We also used 13 *eae*-negative strains.

The presence of *nleA* was determined by PCR with primer V83-for2 in combination with either V83-rev2 or V83-rev3, which is complementary to conserved regions of this gene. Primer V83-rev3 was constructed because it was not possible to amplify a PCR product from all strains with the primer V83-rev2. Total DNA of PCR-negative strains was hybridized with an $nleA_{4795}$ probe to exclude the possibility that negative PCR results were due to variations in primer-binding sites. Only one PCR-negative rabbit O49:H2 strain was detected with the $nleA_{4795}$ probe. Members of the *nleA* gene family were detected in 150 out of 170 strains, and 148 of these isolates carried one of the *eae* alleles β, ε, γ, η, κ, θ, and ζ (Table 2). Interestingly, we were able to detect *nleA* in two *eae*-negative human isolates with serotypes O103:H2 and O111:H−. *nleA* genes were absent in two *eae* ι-positive human strains of serogroup O145. Moreover, *nleA* was not detectable in the EPEC strain E2348/69, which expresses intimin α, in two *eae*-positive strains of serogroup O49, and in three human *eae*-positive O156:H8 isolates (Table 2). *nleA* members were present in the two food isolates but were absent from four *eae*-negative animal isolates and one *eae*-positive animal isolate. Moreover, they were found in 119 out of the 135 human isolates, whereas the 15 *nleA*-negative isolates included 8 *eae*-positive and 8 *eae*-negative *E. coli* strains. In addition, we were able to detect *nleA* genes in all 48 HUS isolates as well as in 40 out of 48 human strains associated with diarrheal disease (Table 2).

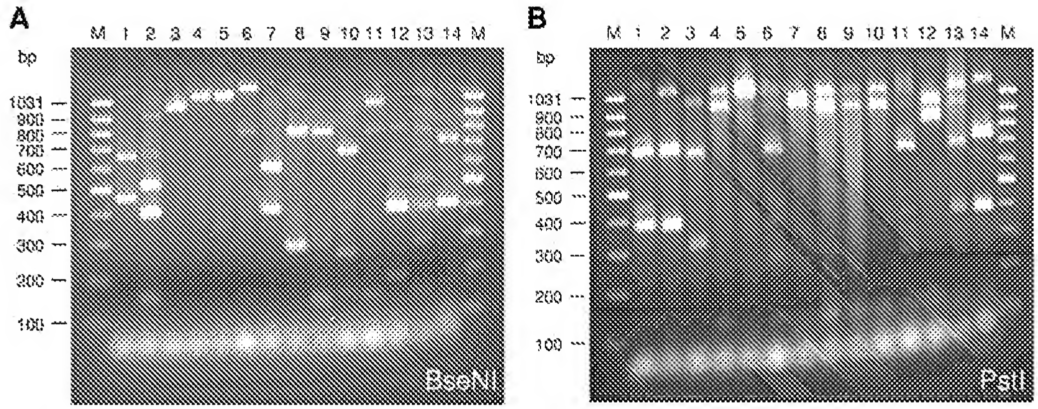

FIG. 1. Agarose gel electrophoresis of selected PCR products of *nleA* variants, each of them separately restricted with BseNI (A) or PstI (B). The PCR products were amplified from the following *E. coli* strains (the variant harbored is named in parentheses): lane 1, 4795/97 (*nleA*$_{4795}$); lane 2, EDL933 (Z6024); lane 3, E22 (the *espI*-like gene); lane 4, 5721/96 (*nleA8-1*); lane 5, 0917/99 (*nleA5*); lane 6, PT272 (*nleA6-1*); lane 7, CB6116 (*nleA7*); lane 8, 3439/00 (*nleA8-1*); lane 9, CF11201 (*nleA8-2*); lane 10, CB6389 (*nleA9*); lane 11, CB8745 (*nleA6-2*); lane 12, CB7690 (*nleA10*); lane 13, 1247/95 (Z6024 and *nleA8-1*); lane 14, DG264/4 (*nleA11*). The molecular weight marker (M) is a GeneRuler 100-bp DNA ladder (Fermentas).

Restriction analyses of the *nleA* variants. The PCR products obtained with primer pairs V83-for2/V83-rev2 and V83-for2/V83-rev3 did not have the same length. Their sizes ranged from 959 bp to 1,168 bp (Table 1). Two restriction enzymes were chosen for differentiation because of the similarity of *nleA*$_{4795}$ and Z6024. Separate restrictions of all PCR products with BseNI (Fig. 1A) and PstI (Fig. 1B) showed 11 different patterns among the 150 PCR products, in addition to the already known variants *nleA*$_{4795}$, Z6024, and the *espI*-like gene of *E. coli*. The expected restriction pattern for *nleA*/*espI* of *C. rodentium* was not observed. After restriction with BseNI, PCR products obtained with strains 5721/96 and 0917/99, depicted in lanes 4 and 5, respectively, showed the same pattern (Fig. 1), whereas different patterns were obtained by using PstI. Furthermore, PCR products of strains 5721/96, 3439/00, CF11201, and CB6389 in lanes 4, 8, 9, and 10 as well as those of strains PT272 and CB8745 in lanes 6 and 11 share the same PstI restriction pattern (Fig. 1), in contrast to their varying BseNI patterns. Moreover, the addition of molecular weights of BseNI and PstI restriction fragments of strain 1247/95 in lane 13 revealed molecular weights that were approximately double the weights of the other strains. Based on this observation and the arrangement of the restriction fragments, we concluded that this PCR product is a mixture of the two *nleA* variants shown in lane 2 and lane 8 (Fig. 1).

Molecular characterization of the *nleA* variants. In order to prove the assumption that each restriction pattern represents an independent *nleA* variant, for each pattern, one strain of each serogroup was selected and the respective PCR product was sequenced. Problems arose during sequencing because of the occurrence of mixed products. This finding, along with the detection of the restriction pattern shown in Fig. 1, lane 13, led to the verification of the hypothesis that some strains may carry more than one copy of *nleA*. Therefore, Southern blot hybridization was performed (Fig. 2).

Two copies of *nleA* variants were detected in most of the *nleA*-positive isolates of serogroups O26, O84, and O156. Furthermore, two copies were detectable in three O49:NM strains, originating from pigs that probably were from the same farm, and one human O103:H2 isolate. All the other *nleA*-positive O49 and O103 strains examined possessed only one copy of the gene. This was also true for all *E. coli* O111, O128, O145, and O157 strains (Fig. 2; Table 2).

DNA fragments were amplified by using primer VarA-for either in combination with VarA-rev, which binds in the region downstream of *nleA*, or in combination with VarA-rev2, which binds at the 3′ end of the gene. For DNA sequencing of PCR products of isolates with two copies of *nleA*, the following strategies were used. If the PCR product appeared as a single band, it could be cloned directly into the pCR2.1-TOPO vector of the TOPO TA cloning kit (Invitrogen). If two PCR products were amplified, the PCR product with the expected size was extracted from a gel prior to sequencing.

DNA sequencing resulted in the identification of 11 new *nleA* variants, termed *nleA1* to *nleA11*, besides the three known variants of pathogenic *E. coli* (Fig. 3). We defined an open reading frame (ORF) with a cutoff value of less than 97% sequence identity at the deduced amino acid level as an individual variant of *nleA*. By sequencing, two *nleA* variants were identified that shared a restriction pattern that was the same as that of the *espI*-like gene. To distinguish variants *nleA1*, *nleA2*, and the *espI*-like gene, PCR products with the primer pair V83-for2/V83-rev2 from all concerned strains were restricted either by Bpu1102I or by CseI. Moreover, many members of variant *nleA8-1* (see below) differ in 1 bp in the recognition site of BseNI, resulting in different restriction patterns. This is shown in lanes 4 and 8 in Fig. 1. To distinguish this variant, *nleA8-1*, from *nleA3* and *nleA4*, which had the restriction pattern shown in lane 4 (Fig. 1), the restriction enzymes BclI, NheI, and SphI were used.

The 15 variants, including *nleA* of *C. rodentium*, revealed sequence identities to each other of between 71% and 96% at the deduced amino acid level. Several variants showed strain-specific differences in the amino acid sequences that were caused by one to three point mutations. These mutations were not taken into consideration in this study. Only the strain-specific differences in the sequence of variant NleA6 and NleA8 were separated by an additional numerical suffix, be-

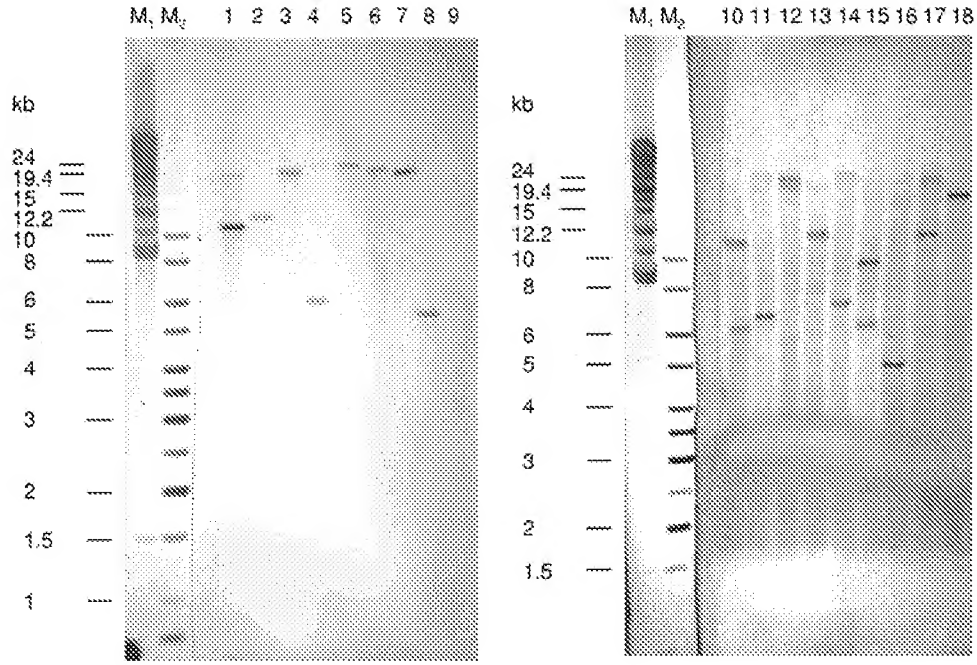

FIG. 2. Southern blot hybridization of different *nleA* variants with an $nleA_{4795}$ probe. The following *E. coli* strains were used (the variant harbored is named in parentheses): lane 1, O84:H4 strain 4795/97 ($nleA_{4795}$); lane 2, O157:H7 strain 0136/96 (Z6024); lane 3, O103:H2 strain 2576/97 (the *espI*-like gene); lane 4, O156:H1 strain LTEC94460 (*nleA1*); lane 5, O145:NM strain 4672/99 (*nleA2*); lane 6, O111:H2 strain 5721/96 (*nleA8-1*); lane 7, O84:H2 strain CB7197 (*nleA4*); lane 8, O145:H28 strain 0917/99 (*nleA5*); lane 9, O127:H6 strain E2348/69 (negative control); lane 10, O156:H25 strain PT272 (*nleA6-1* and *nleA8-2*); lane 11, O84:H$^-$ strain CB6116 (*nleA7* and *nleA8-2*); lane 12, O26:H11 strain 3439/00 (*nleA8-1*); lane 13, O125:H$^-$ strain CF11201 (*nleA8-2*); lane 14, O84:nt strain CB6389 (*nleA8-2* and *nleA9*); lane 15, O156:H25 strain CB8745 (*nleA6-2* and *nleA8-2*); lane 16, O49:NM strain CB7690 (*nleA10*); lane 17, O26:H$^-$ strain 5720/96 (Z6024 and *nleA8-1*); lane 18, O145:H28 strain DG264/4 (*nleA11*). M_1 is the lambda mix marker 19; M_2 is a GeneRuler 1-kb DNA ladder (Fermentas).

cause NleA6-2 possessed an insertion of four amino acids that were absent from NleA6-1, resulting in a deduced protein length of 462 amino acids. On the other hand, NleA8-1 and NleA8-2 varied in 10 amino acids of the C-terminal end (Fig. 3). This difference seems to be associated with specific serogroups (Table 2). Moreover, the length of the 15 deduced proteins varied between 412 and 462 amino acids (Fig. 3). These variations are due to the repeated occurrence of amino acids, in particular of alanine, serine, and threonine, in the middle region of the deduced proteins (Fig. 3). Furthermore, this region includes a putative transmembrane helix. Because of the absence of this region, this putative helix is missing from the variants EspI-like protein, NleA1, NleA2, NleA7, NleA10, and NleA of *C. rodentium* (Fig. 3). Another putative transmembrane helix is located in the C-terminal third of the deduced amino acid sequence. This one is present in all variants described (Fig. 3).

Most of the strains of serogroups O26, O84, and O156, as well as three O49:NM isolates from pigs, probably originating from the same farm, and one O103:H2 strain, harbored two copies of the gene *nleA* (Table 2). Moreover, one copy of *nleA* was disrupted in isolates of serogroups O26, O84, and O156. Sequencing of variant Z6024 of serogroup O26 revealed the deletion of 1 bp, resulting in a truncated, possibly nonfunctional putative protein. This was also the case for *nleA8-1* of the O84:H$^-$ isolate that harbored variants *nleA8-1* and *nleA8-2*. Other strains of serogroup O84 harboring *nleA7* and *nleA8-2* or *nleA9* and *nleA8-2* showed a disrupted ORF of variant *nleA8-2* because of the insertion of the insertion sequence (IS) element ISEc8 651 bp upstream of the 3′ end of the gene. In contrast, 117 bp of the 5′ end of variant *nleA8-2*, present in serogroup O156, was missing. The inserted sequence resembled the region upstream of $nleA_{4795}$ of the prophage BP-4795 and, to some extent, an ISEc8 element. Therefore, 179 bp that was in BP-4795 was missing from this sequence. Furthermore, the first 34 bp of the *espI*-like gene of one O128:H2 pigeon isolate was deleted due to insertion of an IS element, in contrast to other strains of serotype O128:H2 that harbored a complete ORF of the *espI*-like gene. Variant *nleA4* also may encode a truncated protein because of the insertion of 5 bp located 78 bp downstream of the 5′ end of the gene.

Most of the isolates of serogroups associated with severe human disease harbored variant Z6024, *nleA8-1*, or the *espI*-like gene (Table 2). Moreover, only one or three different variants of *nleA* could be detected from the serogroups O26, O157, O103, and O111. In contrast, serogroup O145 appeared to be heterogeneous, with six different variants. Whereas serotype O145:NM was nearly uniform, four variants of *nleA* were detected in the six O145:H28 isolates examined. This also was the case for serogroups O49, O84, and O156. These serogroups harbored five to six different variants, whereas *nleA*-positive O128 strains possessed only the *espI*-like gene. Variants Z6024, *nleA8-1*, and the *espI*-like gene occurred most frequently, followed by variants *nleA8-2* and *nleA2*. In contrast, the other nine variants were detected only in one to six isolates (Table 2). Furthermore, most of the *eae* subtypes β, γ, θ, ζ, and

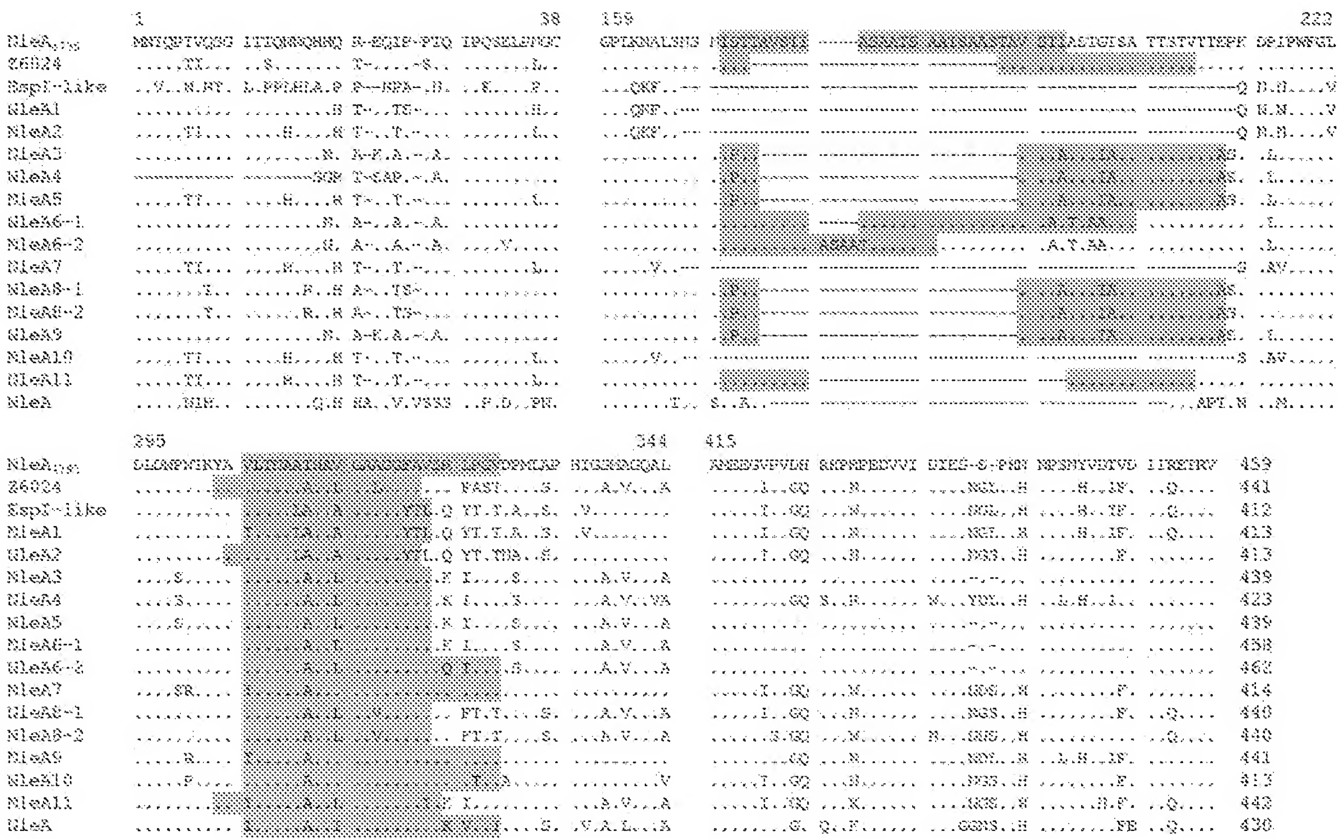

FIG. 3. Alignment of the deduced amino acid sequences of the four known *nleA* variants $NleA_{4795}$, Z6024, the EspI-like protein, and NleA, as well as the newly discovered variants NleA1 to NleA11. Only the N-terminal regions, the two putative transmembrane helices, and the C-terminal regions are shown. The position numbers refer to the sequence of $NleA_{4795}$. Identical amino acids are depicted by dots, and amino acids that are absent from a particular sequence are indicated by dashes. The putative transmembrane helices are labeled in gray.

ε, detected in a larger number of isolates, were associated with four to eight different *nleA* variants (Table 2). Because of the rare occurrence of most of the *nleA* variants as well as the association of the *espI*-like gene, *nleA8-1*, and Z6024 with at least two different *eae* subtypes, a direct correlation between a certain variant of *nleA* and a specific *eae* subtype could not be defined.

Transduction of *nleA* variants. Previously, it was shown that $nleA_{4795}$ and the *espI*-like gene are located in the genome of prophages, which are fully inducible to produce phage particle progeny, whereas Z6024 is located on a cryptic prophage (6, 24, 30). In order to determine whether the newly discovered variants are within intact phages that are able to spread the *nleA*-encoded T3SS effector by horizontal gene transfer, transduction experiments were carried out. A collection of 24 pathogenic *E. coli* strains harboring different *nleA* variants were chosen as donor strains for these experiments (Table 3). *E. coli* C600/pK18 was used as the recipient to prevent the growth of single wild-type cells, which were not detached during preparation of phage lysates. The 11 isolates carrying stx_2 or stx_{2f} that were examined carried inducible Stx2-converting prophages, which were transducible in *E. coli* C600/pK18 (Table 3). All strains harboring stx_2 belonged to serogroups associated with severe human disease, and most of them originated from patients with HUS. Only one strain, the O145:H28 isolate CB4973, also possessed the variant *nleA6-1* in the genome of an inducible prophage. Furthermore, the three isolates 4795/97, 01-08612, and CB6389 of serogroup O84, as well as the O49:NM strain CB7690, obviously harbored functional prophages carrying an *nleA* variant, which could be transduced in the *E. coli* K-12 strain C600/pK18 (data not shown). Strains CB7690, 4795/97, and 01-08612 carried variant *nleA10* or $nleA_{4795}$ in the genome of an inducible bacteriophage. Strain CB6389 possessed an intact prophage harboring variant *nleA9* that is not disrupted by an IS element, and the similar variant *nleA8-2* was located in the genome of this isolate as well. Moreover, each of these three O84 strains exhibited an intact Stx1-converting prophage. This also was the case for the O84:H2 isolate CB7197. However, it was not possible to transduce the variant *nleA4* of this isolate in the *E. coli* strain C600/pK18. No bacteriophages harboring stx_1 or a variant of *nleA* and originating from the other eight stx_1-positive or *stx*-negative strains were detectable after transduction in C600/pK18. Thus, we were able to demonstrate the transduction of five *nleA*-carrying phages with the method described above. However, we cannot exclude completely the possibility that the other 19 analyzed *E. coli* strains also harbor inducible *nleA* phages that could not be detected in this assay.

DISCUSSION

The detection of different variants of the gene *nleA* in 150 out of 170 *E. coli* strains examined shows the widespread oc-

TABLE 3. *E. coli* donor strains used for transduction of phages to the recipient *E. coli* C600/pK18 and transduced genetic traits

Strain	Serotype	Host	Disease[a]	*stx* type	*eae* type	*nleA* type	Transduced trait(s)
1530/99	O26:H11	Human	HUS	2	β	Z6024,[b] *nleA8-1*	stx_2
3439/00	O26:H11	Human	NK	2	β	*nleA8-1*	stx_2
CB7690	O49:NM	Human	HC		κ	*nleA10*	*nleA10*
CB7197	O84:H2	Cattle	NK	1	ζ	*nleA4*	stx_1
03-06145	O84:H2	Human	D	1	ζ	*nleA7, nleA8-2*[c]	
4795/97	O84:H4	Human	D	1	ζ	$nleA_{4795}$	stx_1, $nleA_{4795}$
01-08612	O84:H28	Human	NK	1	ζ	$nleA_{4795}$	stx_1, $nleA_{4795}$
CB8966	O84:H$^-$	Pig	NK		θ	*nleA8-2, nleA8-1*[b]	
CB6389	O84:nt	Human	D	1	ζ	*nleA9, nleA8-2*[c]	stx_1, *nleA9*
UTI	O103:H2	Human	UTI	1	ε	*espI*-like	
2636/97	O103:H$^-$	Human	HUS	2	ε	*espI*-like	stx_2
1639/77	O111:H$^-$	Human	D	1	θ	*nleA8-1*	
1187/00	O111:H$^-$	Human	HUS	1/2		*nleA8-1*	stx_2
T4/97	O128:H2	Pigeon	AS	2f	β	*espI*-like[c]	stx_{2f}
0917/99	O145:H28	Human	D	1	γ	*nleA5*	
4557/99	O145:H28	Human	HUS	2	γ	*nleA3*	stx_2
CB4973	O145:H28	Human	HUS	2	γ	*nleA6-1*	stx_2, *nleA6-1*
DG264/4	O145:H28	Pig	AS		γ	*nleA11*	
4392/97	O145:NM	Human	HUS	2	β	Z6024	stx_2
4672/99	O145:NM	Human	HUS	1/2	γ	*nleA2*	stx_2
CB8104	O145:NM	Human	HUS	2	γ	*nleA2*	stx_2
LTEC94460	O156:H1	Human	AS		ζ	*nleA1*	
PT272	O156:H25	Sheep	NK	1	ζ	*nleA6-1, nleA8-2*[c]	
2492/00	O157:H$^-$	Human	HUS	2	γ	Z6024	stx_2

[a] NK, not known; HC, hemorrhagic colitis; D, diarrhea; UTI, urinary tract infection; AS, asymptomatic.
[b] Variant gene with a 1-bp deletion of either the Z6024 or *nleA8-1* sequence.
[c] The 5′ end of *nleA8-2* or the *espI*-like gene was missing from this gene.

currence of this non-LEE-encoded T3SS effector among pathogenic *E. coli* strains. With the exception of two strains, we could confirm the appearance of *nleA* in association with *eae* as determined by Mundy et al. (26). It has yet to be proven whether the two *eae*-negative isolates are able to secrete NleA or if the gene represents a relic of extensive genetic rearrangement without any known function. Moreover, although the function of the virulence determinant NleA is unknown, the widespread distribution of the encoding gene points to an apparent selective advantage for *E. coli* strains harboring copies of this gene.

DNA sequencing revealed 15 gene variants of different lengths. Interestingly, a region of 4 to 51 deduced amino acids approximately located in the middle of the encoded deduced protein was lacking in some variants. This region, characterized by the predominant occurrence of alanine, serine, and threonine, includes a putative transmembrane helix. Therefore, in variants of *nleA* with a deletion of 36 to 51 amino acids, this helix is missing. Thus, these variants exhibit only one putative transmembrane helix, whereas other members of the *nleA* gene family possess two helices. At present, the role of the number of helices is unknown. Possibly, there are differences in the location or function of the deduced proteins inside eukaryotic cells.

The intimin subtypes seem to be responsible for different host tissue tropisms in the intestine (9). Therefore, they may contribute to the severity of symptoms during a disease caused by EHEC or EPEC. Such an association also was shown for Stx variants. Stx2 often causes more severe disease than those caused by Stx1, whereas differences appear among the heterogeneous members of the Stx2 group (2, 12). Until now, no significant correlation could be determined between the occurrence of a certain *nleA* variant and the appearance of a specific *eae* type, *stx* type, or pathotype. The distribution of different members of the *nleA* gene family seems to be associated more closely with the serogroup of *E. coli* strains. Thus, with the exception of *E. coli* O145 strains, the serogroups associated with more severe disease in humans predominantly contain one or two different *nleA* variants, whereas less important serogroups contain a larger number of variants (Table 2). The majority of O26, O103, O111, and O157 strains harbor Z6024, the *espI*-like gene, or *nleA8-1*. On the other hand, strains of serogroups O49, O84, and O156 harbor a variety of members of the *nleA* gene family. Therefore, these strains may be depicting a pool for genetic rearrangements.

Whereas Z6024 is harbored by the cryptic prophage CP-933P (30), $nleA_{4795}$ and the *espI*-like gene are carried by inducible bacteriophages (6, 24). Transduction experiments also revealed the location of *nleA6-1*, *nleA9*, and *nleA10* in the genome of inducible phages. T3SS effector protein-encoding genes often are present at one end of bacteriophages, presumably a result of incorrect excision during the lytic life cycle. Moreover, the transduction of bacteriophages that carry a variant of the gene *nleA* to a laboratory *E. coli* strain raises the possibility that NleA-converting bacteriophages can be spread by horizontal gene transfer. Some strains harbored two copies of mostly different *nleA* variants. In the majority of the concerned isolates, one of these copies was deleted by an IS element, or some base pairs were missing. This perhaps results from the ability of bacteriophages harboring a member of the *nleA* gene family to infect the *E. coli* strain when parts of another lysogenic phage in the genome of this *E. coli* strain were destroyed by genetic rearrangements. These results indicate a major role for bacteriophages in the distribution of the members of the *nleA* gene family.

Recently, it was shown that *nleA*$_{4795}$ is harbored together with *stx*$_1$ on a single bacteriophage (6). Whether this is the case for other bacteriophages, such as BP-01-08612, BP-CB6389, and BP-CB4973, remains to be elucidated.

Interestingly, the *nleA* variants investigated in this study and those investigated by other authors are linked to phage DNA. This fits with the concept that the specific characteristics of pathogenic *E. coli* strains are located on mobile genetic elements. To elucidate the role of the phage-encoded type III effectors in more detail, further research is needed.

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